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Dscam guides embryonic axons by Netrin-dependent and -independent functions

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Developing axons are attracted to the CNS midline by Netrin proteins and other as yet unidentified signals. Netrin signals are transduced in part by Frazzled (Fra)/DCC receptors. Genetic analysis in *Drosophila* indicates that additional unidentified receptors are needed to mediate the attractive response to Netrin. Analysis of Bolwig's nerve reveals that *Netrin* mutants have a similar phenotype to *Down Syndrome Cell Adhesion Molecule* (*Dscam*) mutants. *Netrin* and *Dscam* mutants display dose sensitive interactions, suggesting that Dscam could act as a Netrin receptor. We show using cell overlay assays that Netrin binds to fly and vertebrate Dscam, and that Dscam binds Netrin with the same affinity as DCC. At the CNS midline, we find that *Dscam* and its paralog *Dscam3* act redundantly to promote midline crossing. Simultaneous genetic knockout of the two *Dscam* genes and the Netrin receptor *fra* produces a midline crossing defect that is stronger than the removal of Netrin proteins, suggesting that Dscam proteins also function in a pathway parallel to Netrins. Additionally, overexpression of *Dscam* in axons that do not normally cross the midline is able to induce ectopic midline crossing, consistent with an attractive receptor function. Our results support the model that Dscam proteins function as attractive receptors for Netrin and also act in parallel to Frazzled/DCC. Furthermore, the results suggest that Dscam proteins have the ability to respond to multiple ligands and act as receptors for an unidentified midline attractive cue. These functions in axon guidance have implications for the pathogenesis of Down Syndrome.

Key words: *Drosophila* genetics, Axon guidance, Body patterning, Cell migration, Central nervous system, Signal transduction

INTRODUCTION

In bilaterally symmetric nervous systems, many axons are guided to the midline of the central nervous system (CNS) by proteins of the Netrin family (Hedgecock et al., 1990; Ishii et al., 1992; Serafini et al., 1994; Kennedy et al., 1994). In *Drosophila*, there are two redundant Netrin genes, *NetA* and *NetB*, and deletions of both lead to greatly reduced axon crossing of the midline (Mitchell et al., 1996; Harris et al., 1996; Brankatschk and Dickson, 2006). Nevertheless, significant numbers of axons persist in orienting towards and crossing the midline in *NetA*/*NetB* mutants, suggesting the existence of additional attractive midline cues (Hummel et al., 1999a; Brankatschk and Dickson, 2006). The *abl* non-receptor tyrosine kinase appears to participate in all midline attractive systems, because null mutants lack all commissures (Grevengoed et al., 2001), and Abl has been shown to function in Netrin signaling (Forsthoefel et al., 2005). The phenotypes of other mutants lacking midline crossing, *commissureless*, *schizo* and *elav/weniger*, have all been traced to effects on the Robo/Slit midline repulsion system (Onel et al., 2004; Keleman et al., 2005; Simionato et al., 2007). Therefore, despite extensive genetic screens (Seeger et al., 1993; Hummel et al., 1999b), components of the missing midline attractive system have yet to be identified and represent a major challenge for our understanding of the formation of the *Drosophila* ventral nerve cord.

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There is also evidence for an unidentified attractive Netrin receptor. The work that identified DCC (Deleted in Colorectal Cancer) as a Netrin receptor noted that some DCC-positive axons do not show any responses to Netrin, and postulated that the presence of a co-receptor might be required (Keino-Masu et al., 1996). The *C. elegans* DCC homolog UNC-40 generally has mutant phenotypes that are less severe than UNC-6 (Netrin) mutants, suggesting the existence of a second pathway to respond to UNC-6 (Chan et al., 1996). In *Drosophila*, a single DCC family member, *frazzled* (*fra*), is present (Kolodziej et al., 1996). The *fra* (DCC) CNS phenotype is similar, but not identical to *NetA*/*NetB* deletions, as might be expected (Brankatschk and Dickson, 2006; Garbe and Bashaw, 2007; Garbe et al., 2007). In addition, for both migrating salivary glands and Netrin-responsive motor axons, the *frazzled* (*fra*) mutant phenotypes are of lower penetrance than those of *NetA*/*NetB* deletions (Kolesnikov and Beckendorf, 2005; Winberg et al., 1998; Labrador et al., 2005). Finally, two studies have provided convincing data that *fra* plays a non-autonomous role in axon guidance. In the embryo, the pioneer axon dMP2 has an altered trajectory in *fra* mutants; rescue of the mutant phenotype is not achieved by expression of *fra* in dMP2 alone, but requires expression by the cells encountered by the dMP2 axon (Hiramoto et al., 2000). In retinal projections, loss of axonal *fra* has little effect on their pathfinding, but loss of *fra* in the target tissue, the lamina, cause dramatic errors (Gong et al., 1999). In each case, Fra is thought to present Netrin to an unidentified receptor on the navigating axons.

The *Drosophila Down Syndrome Cell Adhesion Molecule* (*Dscam*) gene has been the focus of considerable attention owing to the potential to encode 38,016 distinct protein isoforms through alternative splicing (Schmucker et al., 2000; Wojtowitz et al., 2004; Zipursky et al., 2006). However, there is minimal alternative splicing in vertebrate *Dscam* genes, and in the three other *Drosophila Dscam* genes (Yamakawa et al., 1998; Agarwala

et al., 2001; Crayton et al., 2006). Given the evolutionary conservation of these molecules, this suggests that there is an important *Dscam* function that does not depend on molecular diversity. Genetic evidence in *Drosophila* also supports a diversity-independent function (Chen et al., 2006; Hattori et al., 2007). Like *Drosophila* Dscam, vertebrate Dscam proteins are capable of mediating homophilic cell adhesion (Agarwala et al., 2000; Agarwala et al., 2001). Knockdown of *Dscam* function in zebrafish leads to impaired cell movement, whereas perturbation of *Dscam* in the planarian disrupts cell migration, axon outgrowth and fasciculation (Yimlamai et al., 2005; Fusaoka et al., 2006). The diversity of these phenotypes, coupled with the previously noted similarity of Dscam to other axon guidance receptors (Yamakawa et al., 1998), suggests that the primary Dscam function could be to respond to extracellular, perhaps diffusible, ligands.

We found that *Netrin* mutants have similar phenotypes to *Dscam* mutants in Bolwig's nerve (the larval photoreceptor organ), suggesting that Dscam could function as a Netrin receptor. A physical interaction was confirmed in vitro using cell overlay assays. We also uncovered a subtle axon guidance defect in embryos mutant for one of the three additional *Dscam* genes in *Drosophila*: *Dscam3* (*CG31190*) (Millard et al., 2007). Genetic interactions between *Dscam*, *Dscam3* and the *abl* tyrosine kinase indicated a role for the *Dscam* genes in midline crossing. Genetic interactions with the Netrin receptor *fra* suggested that the Dscam proteins function in a parallel pathway to Netrin signaling. We favor a novel model in which Dscam proteins are required for the transduction of several different axon guidance and cell migration cues, most probably through combinatorial association with other receptors.

MATERIALS AND METHODS

Genetics

The piggybac line *c02826* has two insertions (Thibault et al., 2004), one in *Dscam3* and a second in *CG7433*, a 4-aminobutyrate (GABA) aminotransferase, which was removed by recombination. Interestingly, the parental double insert was not recovered in the F2 generation, suggesting that the double mutant is less viable than either single mutant alone, possibly suggesting a synergistic interaction between the genes. The *CG7433* insert does not appear to affect the CNS phenotypes. The *f01683 Dscam3* allele is a lethal chromosome, owing to at least two background lethal mutations, and may only mildly disrupt *Dscam3*. The piggybac transposons were generated in a w^- isogenic background, which has significant effects on BN and motoneurons, so was not used as a control. *fra* alleles were obtained from P. Kolodziej and G. Tear. abl^1, abl^4, *Dscam* alleles and *Df(1)NP5* were obtained from the Bloomington Stock Center. $NetA,B^{\Delta}$ was obtained from B. Dickson. In our crosses, we found the TM6 balancer increased phenotypic penetrance, and we suspect that the presence of *Tb* has a detrimental effect on viability, and appears to exert a maternal effect on the CNS phenotypes. Viable combinations of Dscam proteins frequently show larval, pupal and adult phenotypes resembling *Tb*. To rule out *Tb* effects on axon guidance, all phenotypes were confirmed in the absence of balancer chromosomes by out-crossing to wild-type chromosomes, and by crossing the F1 progeny that lacked balancers.

DNA constructs

Drosophila Dscam was PCR amplified from the LP cDNA library (Berkeley Drosophila Genome Project) and cloned into pcDNA3-V5-His-Topo (Invitrogen). The *Dscam* splice isoform used in this work was 1-30-30-2. Human *Dscam* in pcDNA3 was a gift from W. Li and K.-L. Guan. The pGNET-myc chick *Netrin-1* and pcDNA3-HA-ratDCC constructs were gifts from M. Tessier-Lavigne. *Drosophila NetB* was PCR amplified from a cDNA clone (gift of G. Bashaw), and subcloned into pcDNA3-myc-His (Invitrogen). Sema3A and Sema3F expression constructs were gifts from H. Cheng.

Immunohistochemistry

Drosophila immunohistochemistry was performed as described by Patel (Patel, 1994). Mouse embryo immunohistochemistry was performed as follows: CD-1 mice were crossed and the day of the plug was designated E0.5. On the specified embryonic day, embryos were dissected free of the uterus in 0.1 M phosphate buffer. The embryos were then fixed in 4% paraformaldehyde in 0.1 M phosphate buffer for 1 hour on ice, embedded in gelatin, frozen on a 2-methylbutane slurry and sectioned on a Leica cryotome. The sections were blocked for 1 hour in 5% goat serum in PBS (phosphate-buffered saline) containing 0.1% Tween-20. Both anti-βIII-tubulin (Covance MMS-435P) and anti-hDSCAM (gift from K. Guan) were applied at 1:1000 dilutions overnight. Cy2- or Cy3-conjugated secondary antibodies (Jackson) were applied at a 1:200 dilution for 1 hour. All washes were carried out with PBS containing 0.1% Tween-20.

Cell overlay assays

COS-7 cells at 80% confluency were transfected with DNA expression constructs using Lipofectamine 2000 (Invitrogen), according to manufacturer's instructions (200 μg DNA per 100 μl). Approximately 40 hours post-transfection, the supernatant was removed from receptor-expressing cells and replaced with supernatant containing epitope-tagged

Table 1. Quantification of axon guidance defects seen in Bolwig's nerve

Genotype	Total counted	% Mild defects	% Severe defects	% Total defects	
Oregon R (wild type)	50	2	2	4	
$Dscam^{P/P}$	51	15.6	11.8	27.5	
$NetA,B^{NP5}/Y$	52	40	50	90	
$NetA,B^{\Delta}/Y$	52	36.5	59.6	96.2	
fra^3/fra^4	50	16	10	26	
$Dscam^{P/P}$ $fra^{4/4}$	54	28	39	67	
$Dscam^{P/P}$ $fra^{4/4}$ $Dscam3^{1/1}$	50	12	46	58	
$NetA,B^{\Delta}/+$	54	3.7	0	3.7	
$NetA,B^{NP5}/+$	50	4	2	6	
$Dscam^{P}/+$	64	4.7	0	4.7	
$fra^4/+$	60	8.3	1.7	10	
$NetA,B^{NP5}/+$; $fra^4/+$	70	14.3	15.7	30	***
$Dscam^{P}$ +/fra^4 +	40	17.5	5	22.5	***
$NetA,B^{NP5}/+$;$Dscam^{P}/+$	100	15	23	38	***
$NetA,B^{\Delta}/+$; $Dscam^{P}/+$	50	10	24	34	***

Bolwig's nerve was scored for defects in axon guidance and fasciculation in homozygous and hemizygous combinations (top four rows) and in heterozygous combinations (bottom seven rows). We subdivided the phenotypes observed into mild and severe; a phenotype was severe if more than one axon bundle defasciculated from BN, if the defasciculation occurred early in the trajectory or if the trajectory was dramatically altered from wild type. Outcrossing to wild type was crucial to the trans-heterozygote analysis, as the presence of balancer chromosomes was found to enhance BN phenotypes. The $NetA,B^{\Delta}$, $NetA,B^{NP5}$, $Dscam^{P}$ and fra^4 trans-heterozygous combinations were found by logical regression to increase the percentage of defects beyond purely additive effects (***P<0.0001).

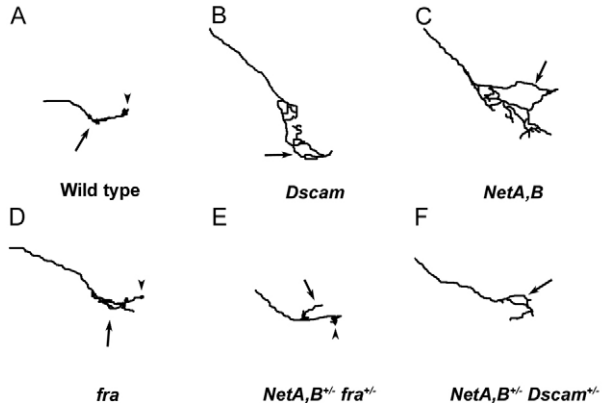

Fig. 1. *Dscam* and *Netrin* phenotypes in Bolwig's nerve. The axons of Bolwig's Nerve (BN; the larval photoreceptor organ) in stage 17 embryos were visualized with monoclonal antibody 22c10 and trajectories traced digitally. The axons shown are growing along the external ventral side of the brain hemisphere. See Fig. S1 in the supplementary material for the original figure. (**A**) Wild-type BN showing tightly bundled axons and growth cones (arrowhead) as they grow around the brain hemisphere making a characteristic turn (arrow). (**B**) *Dscam* homozygous embryo showing defasciculation of BN, and refasciculation to form a small loop (arrow). (**C**) *NetA,B* hemizygote showing extensive defasciculation of BN and refasciculation to form a large loop (arrow). (**D**) *fra*^{3} homozygote showing defasciculation (arrow) and separation of a growth cone from a club-like structure (arrowhead). (**E**) *Dscam fra*/+ + trans-heterozygous embryo showing defasciculation (arrow) and a club like structure (arrowhead). (**F**) *NetA,B*/+; *Dscam*/+ trans-heterozygote showing a large loop (arrow).

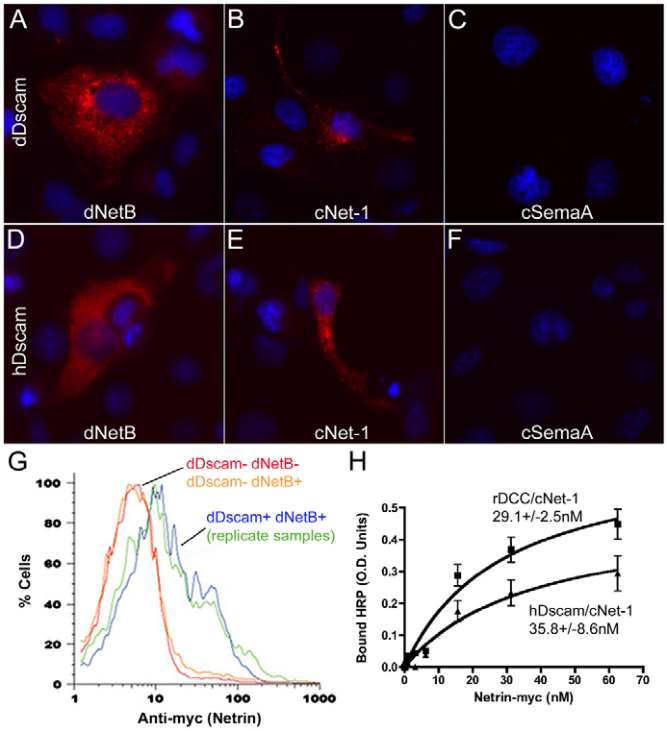

Fig. 2. Dscam and Netrin proteins physically associate in cell overlay assays. COS-7 cells were transfected with plasmids encoding *Drosophila* (A-C) or human (D-F) Dscam. The cells were incubated with medium containing secreted myc epitope tagged *Drosophila* Netrin B (dNetB; A,D), chick Netrin-1 (cNet-1; B,E) or chick Semaphorin A (cSemaA; C,F). After washing, the cells were stained with an anti-myc antibody (red) and counterstained with DAPI (blue). (**A**) dNetB binding to a cell expressing dDscam. (**B**) cNet-1 binding to a cell expressing dDscam. (**C**) cSemaA does not bind to cells expressing dDscam. (**D**) dNetB binding to a cell expressing hDscamE. (**E**) cNet-1 binding to cells expressing hDscam. (**F**) cSemaA does not bind to cells expressing hDscam. (**G**) Flow cytometry quantitation of the binding of dNetB to COS-7 cells expressing dDscam (blue and green lines). Control cells not expressing dDscam and not incubated with dNetB (red line), and control cells incubated with dNetB (orange). The peaks between the controls and the samples are separate, but the shoulders overlap owing to low transfection efficiency of the dDscam construct. (**H**) The binding of human DSCAM and rat DCC to increasing concentrations of myc tagged Netrin were measured using a HRP-conjugated secondary antibody. Scatchard analysis of the data gives dissociation constants of 29.1±2.5 nM for rat DCC/Netrin-myc and 35.8±8.6 nM for DSCAM/Netrin-myc. This difference is not statistically significant (P=0.19, t-test).

ligand in the presence of 0.1% sodium azide. Cells were incubated at room temperature for 45 minutes before rinsing three times in 1×PBS and proceeding with antibody labeling. After rinsing, cells were fixed for 15 minutes in 4% paraformaldehyde with 0.1% Tween-20. The cells were blocked in 5% heat-denatured normal goat serum in 1×PBS plus 0.1% Tween-20 for 15 minutes. After blocking, the cells were incubated with primary antibodies diluted in 5% heat-denatured normal goat serum plus 1×PBS for 45 minutes [rabbit polyclonal anti-human DSCAM 1:1000 (Li and Guan, 2004); rabbit polyclonal anti-drosophila DSCAM 1:500 (Schmucker et al., 2000); anti-myc mouse monoclonal 1:200 (Calbiochem)]. Cells were then rinsed three times in 1×PBS plus 0.1% Tween-20. Secondary antibodies were diluted in 5% heat denatured goat serum in 1×PBS, then added to the cells and incubated for 30 minutes at room temperature (Jackson Labs Cy2 anti-rabbit 1:200 and Cy3 anti-mouse 1:200). The cells were subsequently washed in 1×PBS plus 0.1% Tween-20, followed by 1 minute incubation in 4′,6-diamidino-2-phenylindole (DAPI) to stain the nuclei (Molecular Probes). The cells were then washed in 1×PBS and mounted in FluorSave (Calbiochem).

Binding affinity

For binding affinity determination, COS cells were plated in 24-well plates at 50,000 cells/well and cultured in DMEM + 10% FBS + Pen/Strep. The next day, cells were transfected with 1 μg of pCDNA3.1, pCDNA3-HA-ratDCC (Hong et al., 1999) or pCDNA3-humanDSCAM (Li and Guan, 2004) using Lipofectamine 2000 (Invitrogen). One day later, the culture media was replaced with increasing concentrations of Netrin-myc protein in DMEM containing 10% FBS and 0.1% azide and incubated for 1 hour. The amount of Netrin-myc protein was quantified prior to use by western blot comparison with a standard curve of purified recombinant chicken Netrin-myc (Serafini et al., 1994). The cells were washed, fixed with 4% PFA for 5 minutes, and blocked using PBS containing 0.1% triton and 10% goat serum. Detection of the bound Netrin-myc protein was carried out using an anti-myc primary antibody (9E10) and a goat anti-mouse horseradish peroxydase (HRP) secondary antibody (Jackson) in PBS containing 0.1% triton and 1% goat serum. HRP activity was detected using SigmaFast OPD (*o*-Phenylenediamine dihydrochloride) peroxydase substrate (Sigma) and read using a microplate spectrophotometer.

Statistical analysis

For determining the significance of trans-heterozygote combinations, the PROC GENMOD procedure in SAS software version 9.12 was used to fit a binary logistic regression model. The ESTIMATE option was used to estimate the differences between the linear combinations of the specific trans-heterozygote combinations. A chi-square test was performed to test whether the differences between the specified contrasts are statistically significant at 5% level. The SP1 neuron data were analyzed by fitting a Poisson model using the PROC GLIMMIX procedure in SAS software. Scatchard analysis was performed on four independent experiments using Prism 4 (GraphPad Software), and statistical significance was assessed using the t-test.

Table 2. Quantification of midline crossing by BP102 positive commissural axons

	Anterior commissure		Posterior commissure		Both commissures		
Genotype	Absent	Thin	Absent	Thin	Other	Normal	*n*
OregonR	0%	0%	0%	0%	1%	99%	104
NetA,B^{NP5}	2%	4%	8%	15%	10%	75%	131
NetA,B$^{T9\text{-}B118}$	0%	4%	19%	20%	14%	64%	118
NetA,B$^{\Delta}$	2%	12%	2%	15%	36%	50%	110
fra$^{3/4}$	2%	4%	3%	8%	8%	68%	110
fra^{Z965}	1%	0%	4%	18%	26%	62%	114
abl^{4}	1%	2%	1%	1%	50%	48%	110
Dscam^{P}	0%	0%	0%	0%	30%	70%	99
Dscam3^{1}	0%	0%	0%	0%	44%	56%	104
Dscam^{P} Dscam3^{1}	0%	1%	0%	0%	84%	16%	97
Dscam^{P} fra^{4}	9%	24%	5%	39%	51%	10%	110
fra$^{3/4}$ Dscam3^{1}	2%	3%	1%	9%	48%	37%	132
Dscam^{P} abl^{4}	23%	33%	65%	26%	19%	7%	108
fra^{4} abl^{4}	14%	24%	16%	24%	19%	2%	88
Dscam3^{1} abl^{4}	1%	0%	2%	0%	85%	13%	93
Dscam^{P} fra^{4} Dscam3^{1}	39%	51%	36%	55%	5%	5%	99
Dscam^{P} fra^{4} abl^{4}	98%	2%	100%	0%	0%	0%	110

The anterior and posterior commissures were scored for decreased midline crossing in the homozygous or hemizygous genotypes shown. Segments were also scored for failure to correctly separate the commissures so that two midline glia were visible ('other'). The percentages for the 'absent' and 'thin' columns are for their relevant commissure only; the 'normal' and 'other' categories have been combined as a percentage for both commissures together. The number of segments scored is *n*. OregonR is a wild-type strain. *NetA,B^{NP5}*, *NetA,B$^{T9\text{-}B118}$* and *NetA,B$^{\Delta}$* are deficiencies that delete both *NetA* and *NetB*; *NetA,B$^{\Delta}$* deletes only the netrin genes (Brankatschk and Dickson, 2006). *fra$^{3/4}$* is a protein null combination of alleles from different genetic backgrounds (Kolodziej et al., 1996). *Dscam^{P}* is a P element transposon insertion (Schmucker et al., 2000). *Dscam3^{1}* is a piggybac transposon insertion, *c02826*, into *Dscam3*. Percentages were rounded up to the nearest whole number.

RESULTS

Netrin mutants resemble *Dscam* mutants in Bolwig's nerve

The *Drosophila* larval photoreceptor organ, Bolwig's nerve (BN), extends a bundle of twelve axons to targets in the brain hemispheres, with axons making two stereotyped turns at choice points on the surface of the brain lobe (Schmucker et al., 1997; Schmucker et al., 2000). Early in development, BN axons contact the optic lobe anlagen (OLA) and remain in contact with the OLA during the morphogenetic movements of head involution. After the completion of head involution, BN axon pathfinding resumes, and genetic evidence strongly suggests that axon guidance errors in BN appear not just as altered trajectories of the nerve as a whole, but also as defasciculation of the constituent axons (Schmucker et al., 1997). We examined embryos lacking both *Drosophila* netrin genes (*NetA,B*), and found defasciculation and altered trajectories in 90-96% of embryos (Fig. 1C; Table 1). The nearly complete penetrance of *Netrin* mutants in BN suggests they are the primary guidance cue for BN as it grows around the brain hemisphere. The closest source of *Netrin* expression to BN is the CNS midline and the mesoderm, suggesting that netrin genes act as long-range chemoattractants for BN, much as the migrating salivary glands are guided (Kolesnikov and Beckendorf, 2005).

The *NetA,B* mutant phenotype in BN closely resembles that of *Dscam* mutants, which have been extensively characterized in axon guidance of BN (Schmucker et al., 2000). Phenotypes of the Netrin receptor *fra* in BN have not previously been described. *Dscam* and *fra* mutants both displayed similar severity of BN errors, but with much lower penetrance when compared with the *Netrin* mutant (Table 1). *Dscam fra* double mutants showed an increased penetrance, but still less than that of *NetA,B* mutants alone. Removal of *Dscam3* did not increase the penetrance of *Dscam fra* double mutants, suggesting additional unidentified receptors are involved in the guidance of BN. The phenotypic similarities observed for *Dscam*, *fra* and *NetA,B* suggested the genes might be functioning in the same pathway. To genetically test this, dosage-sensitive interactions between axon guidance genes can be informative (Bashaw et al., 2000; Schmucker et al., 2000). *NetA,B$^{\Delta}$*/+, *NetA,B^{NP5}*/+ and *Dscam^{P}*/+ exhibit BN defects of 3.7%, 6% and 4.7%, respectively (Table 1). Removing one copy of each *Netrin* and *Dscam* simultaneously (trans-heterozygote) increased the penetrance of BN defects to 34% and 38% depending on the *Netrin* deficiency used (Fig. 1F, Table 1). This is an increase of 11.8% (*NetA,B$^{\Delta}$*) or 13.2% (*NetA,B^{NP5}*) over the additive effects of the individual heterozygous phenotypes ($P<0.0001$). This genetic interaction suggests that netrin genes and *Dscam* could function together. *fra* and netrin genes also displayed a dose-sensitive genetic interaction, showing an increase of 4.5% of defects ($P<0.0001$). It is notable that such trans-heterozygote interactions between *fra* and netrin genes have not been reported in the CNS. As DCC (Fra) can physically interact with other guidance receptors, we looked at *Dscam fra* trans-heterozygotes and found a 10.2% increase in BN defects, suggesting they act in the same pathway in vivo.

Dscam and Netrin proteins physically interact

The individual mutant and trans-heterozygote analysis of BN strongly suggested that Dscam might be acting as a Netrin receptor. We tested whether Dscam could bind Netrin proteins using a cell overlay assay (Keino-Masu et al., 1996). COS-7 cells were transfected with constructs expressing either *Drosophila* or human Dscam genes (*Dscam*, *DSCAM*). Then epitope-tagged *Drosophila* NetrinB (NetB) or chick Netrin 1 (cNetrin-1) (Serafini et al., 1994) proteins were incubated with the Dscam-expressing cells. Netrin proteins were detected by immunohistochemistry with antibodies directed against the myc epitope encoded by the expression vector (Fig. 2). Neither Netrin bound to mock-transfected COS-7 cells, but both NetB and cNetrin-1 showed specific binding to cells expressing either *Dscam* or *DSCAM*. Supernatant containing mouse Sema3A or Sema3F was used as control (Cheng et al., 2001). Neither Sema bound to *Dscam*, and both showed very low binding to *DSCAM*. Cell-surface expression of *DSCAM* was confirmed with an anti-DSCAM antibody (Li and Guan, 2004), and the *Drosophila* Dscam was detected by a V5 epitope at the C terminus. Both Dscam proteins also caused the COS-7 cells to round up and become less

adherent as was previously observed for *DSCAM* (Li and Guan, 2004). We also observed Dscam+ COS-7 cells adhering to adjacent cells, and Netrin co-localizing to the site of adhesion, suggesting that the receptor and cell adhesion functions of Dscam are not mutually exclusive. *Drosophila* Dscam expression in COS-7 cells was greatly improved by the addition of C-terminus epitope tags, but transfection efficiency for this construct still remained low. We quantified binding of NetB to Dscam by flow cytometry (Fig. 2G); the low transfection efficiency of the Dscam construct leads to an incomplete separation of the control and experimental peaks, but clearly shows an increase in NetB binding between control and Dscam-expressing cells, confirming a physical association between NetB and Dscam. To quantify the Netrin binding, we compared the dissociation constants (K_d) for cNetrin-1 binding to human DSCAM and to rat (r) DCC (Fig. 2H). The K_d values were determined to be 29.1±2.5 nM for rDCC/netrin-myc and 35.8±8.6 nM for DSCAM/Netrin-myc, indicating that DCC and Dscam bind Netrin with similar affinity. We conclude that Dscam proteins are evolutionarily conserved Netrin-binding proteins.

Dscam proteins affect CNS midline axon crossing

The presence of a cytoplasmic 'PYAT' motif in all four *Drosophila Dscam* genes first brought them to our attention, as the site is similar to the Robo CC1 'PTPYATT' Abl phosphorylation site (Bashaw et al., 2000). The non-receptor tyrosine kinase, Abl, plays an important role in axon guidance (Lanier and Gertler, 2000). Dscam protein is present on all CNS axons (Schmucker et al., 2000) and *Dscam3* has a similar mRNA pattern, so we examined genetic interactions between *abl*, *Dscam* and *Dscam3* for effects on CNS axons. Removal of only the zygotic component of *abl* leads to subtle phenotypes in CNS axons (Fig. 3F). Similarly, both *Dscam* and *Dscam3* display very subtle disorganization of the CNS axon scaffold (Fig. 3D,E). However, double mutants of *Dscam* and zygotic *abl* display dramatic midline crossing defects (Fig. 3H). Removal of *Dscam3* and zygotic *abl* leads to a subtle increase in disorganization of the axon scaffold (Fig. 3G; Table 2). Finally, as the Netrin receptor *fra* and *abl* have been shown to interact genetically (Forsthoefel et al., 2005) (see Fig. S2 in the supplementary material), we examined embryos triply mutant for *Dscam*, *fra* and zygotic *abl*. This triple mutant has a dramatic reduction in the number of axons crossing the CNS midline (Fig. 3I). A double mutant between *Dscam* and *Dscam3* had little effect on midline crossing, although the symmetry of the axon scaffold was disrupted in an additive fashion (Fig. 3J; Table 2). The strong interaction between *Dscam*, *fra* and *abl* prompted us to examine *Dscam fra* double mutants, which show a strong reduction in midline crossing (Fig. 3K). Embryos lacking *fra*, *Dscam* and *Dscam3* show an even stronger reduction (Fig. 3L). As these double and triple mutant phenotypes are far more severe than either *Netrin* or *fra* mutants alone, they suggest that Dscam genes are functioning in a parallel pathway to Netrin-mediated attraction. However, this does not rule out redundancy of Dscam genes within multiple signaling pathways. For example, *abl* appears to operate in both Netrin-dependent and -independent midline crossing pathways (Forsthoefel et al., 2005).

Analysis of identified commissural axons in *Dscam* mutant combinations

The anterior and posterior commissures in *Drosophila* are pioneered independently, in a process that is crucially dependent on cell migration (Goodman and Doe, 1993). The earliest identified commissural axon known is the SP1 neuron, which crosses the

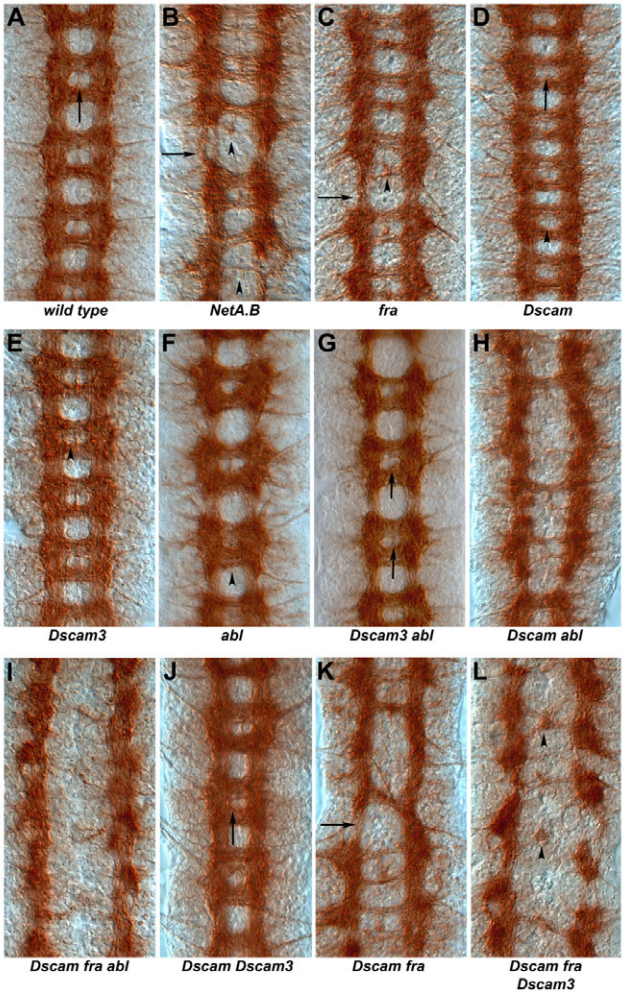

Fig. 3. *Dscam* mutants and *abl* genetically interact in CNS axon guidance. Ventral nerve cords from stage 16 embryos stained with monoclonal antibody BP102 to visualize the CNS axon scaffold with the characteristic pattern of segmentally repeated commissures crossing the CNS midline, and linked by longitudinal tracts. The anterior and posterior commissures are separated by two midline glia, which are separated by a centrally projecting axon (arrow in A). (**A**) Wild-type CNS axon scaffold. (**B**) Embryos lacking *NetA,B* (NP5 deficiency) displaying thin and absent commissures (arrowheads), and irregular longitudinal tracts (arrow). (**C**) *fra* homozygote showing thin or absent commissures (arrowhead) and altered longitudinal tracts (arrow). (**D**) *Dscam* homozygote, which closely resembles wild type. The arrow indicates two midline glia that have not completely separated the commissures, although both are visible. The arrowhead indicates another incomplete separation. The commissures and longitudinal tracts are otherwise unaffected. (**E**) *Dscam3* homozygote. The arrowhead indicates two midline glia that have not fully separated the commissures (subtle). (**F**) Embryo lacking zygotic *abl* function. The arrowhead indicates a failure to separate the anterior and posterior commissures. (**G**) *Dscam3 abl* homozygote, showing several segments in which the commissures have failed to separate (arrows). (**H**) *Dscam abl* homozygote, displaying severe disruption of the commissures. (**I**) Embryo mutant for *Dscam*, *fra* and *abl* showing an absence of commissures. (**J**) *Dscam Dscam3* double mutant showing disrupted separation of commissures (arrow), and an overall irregularity to the CNS axon scaffold. (**K**) *Dscam fra* homozygote displaying greatly reduced or absent commissures, and disrupted longitudinal tracts (arrow). (**L**) *Dscam fra Dscam3* triple mutant homozygote with very few axons crossing the midline, and significantly disrupted longitudinal axon tracts. The residual staining at the midline appears to originate from neurons whose cell bodies reside in the midline (VUMs; arrowheads).

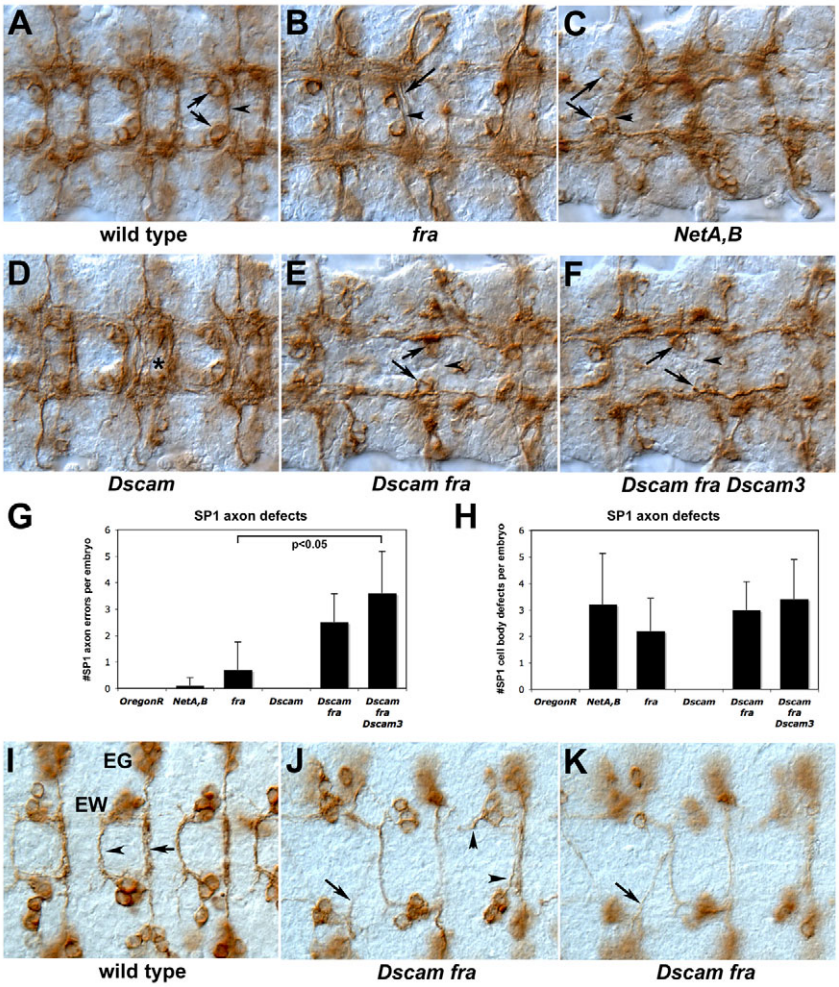

Fig. 4. Analysis of identified commissural axons in *Dscam* mutant combinations. Embryos stained with anti-Connectin antibody (A-F) or the *egl-GAL4; UAStaulacZ* reporter (I-K). Connectin staining highlights the SP1 cell body (arrows) and commissural axon, which crosses the midline in the anterior commissure (arrowheads). All embryos are late stage 15 or early 16, with anterior towards the left. (**A**) Wild-type embryo. Note the regular position of the SP1 cell bodies (arrows) and the commissural axons crossing the midline in the anterior commissure (arrowhead). (**B**) *fra* mutant showing altered SP1 cell body position (arrow), but intact SP1 axon (arrowhead). The posterior commissure is clearly disrupted in the same segment, as is the adjacent longitudinal. (**C**) *NetA,B* mutant showing normal and disrupted SP1 cell body migration (arrows) and missing commissural axons (arrowhead). (**D**) *Dscam* mutant embryo, which is nearly identical to wild type, except for some Connectin-positive axons inappropriately crossing the midline between the two commissures (asterisk). (**E**) *Dscam fra* double mutant displaying missing commissural axons (arrowhead) between the two SP1 cell bodies (arrows; one slightly out of focus). (**F**) *Dscam fra Dscam3* triple mutant showing missing commissural axons (arrowhead) between mispositioned SP1 cell bodies (arrows). (**G**,**H**) Graphs of SP1 axon midline crossing defects (G) and SP1 cell body migration defects (H) in mutant combinations. Abdominal segments1-7 were scored for ten embryos for each genotype. Statistical significance was assessed by fitting the data to a Poisson distribution. (G) SP1 axon defects. *Dscam fra Dscam3* embryos are statistically different from *fra* mutant embryos (P=0.013), indicating that Dscam proteins enhance the *fra* SP1 axon phenotype. *Dscam fra Dscam3* embryos were statistically different from all other mutants (P<0.01), except *Dscam fra*. *Dscam fra* embryos were statistically different from OregonR, *NetA,B* and *Dscam* embryos (P<0.05), but were not found to be significantly different from *fra* alone. (H) Graph of SP1 cell body migration defects. The *Dscam* and OregonR genotypes are statistically different from all other genotypes, except each other (P<0.05). There is no statistical difference between the *NetA,B, fra, Dscam fra* and *Dscam fra Dscam3* phenotypes, indicating that *Dscam* and *Dscam3* do not enhance the *fra* SP1 cell migration defect. (**I**) Wild-type embryo showing expression of the *egl-GAL4;UAS-tau-lacZ* transgene. The EW axons cross the midline in the posterior commissure (arrowhead), and the EG cross in the anterior commissure (arrow). (**J**,**K**) Two focal planes of one embryo, mutant for *Dscam fra*. The arrows indicate an EG axon bundle, crossing the midline in an adjacent posterior commissure in response to a missing anterior commissure (lower). The arrowheads indicate EW axons either failing to cross the midline (upper) or crossing the midline in an adjacent posterior commissure in response to an absent anterior commissure. The *egl-GAL4;UAS-tau-lacZ* transgenes do not switch on *lacZ* in all *egl*-positive cells in each segment; we have found no evidence of altered cell fate or missing cells in *Dscam* mutant combinations.

midline in the anterior commissure and helps to pioneer the longitudinal connectives after crossing (Mellerick and Modica, 2002). The SP1 neuron migrates towards the midline, to occupy positions just anterior to the future anterior commissure; the SP1 axons cross the midline, and grow around the contralateral cell body to join a longitudinal pathway (Fig. 4). We used the anti-connectin antibody C1.427 to visualize the SP1 cell body and axon (Meadows et al., 1994), in various combinations of *Dscam* and *fra* (Fig. 4).

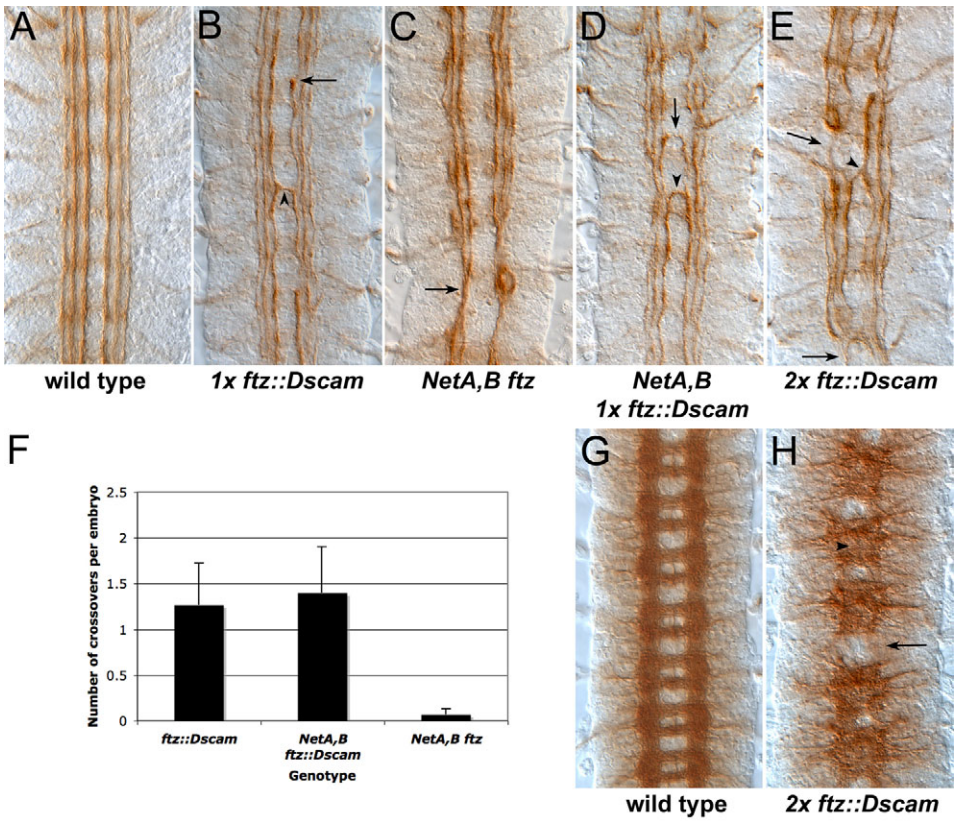

Fig. 5. *Dscam* misexpression phenotypes. Stage 17 embryos stained with anti-Fas2 mAb 1D4 (A-E) and stage 16 embryos stained with MAb BP102 (G,H). (**A**) Wild-type embryo in which Fas2-positive longitudinal fascicles do not cross the midline. (**B**) Embryo carrying one copy each of the *ftz$_{ng}$GAL4* and *UAS-Dscam1-30-30-2* transgenes showing a Fas2-positive fascicle crossing the midline (arrowhead) and also stalled axons (arrow). (**C**) Embryo mutant for *NetA,B* and carrying the *ftz$_{ng}$-GAL4* transgene, showing a collapse of the longitudinal fascicles into one fascicle (arrow) but no ectopic midline crossing. (**D**) Embryo mutant for *NetA,B* and carrying one copy of the *ftz$_{ng}$-GAL4* and *UAS-Dscam1-30-30-2* transgenes, in which thick (arrowhead) and thin (arrow) fascicles can be seen ectopically crossing the midline. The staining below the thick fascicle is a combination of staining in the transverse nerve and residual staining in cell bodies above and below the plane of focus of the longitudinal axons, respectively. (**E**) Embryo carrying two copies each (based on proportion of embryos displaying this phenotype) of the *ftz$_{ng}$GAL4* and *UAS-Dscam1-30-30-2* transgenes showing disrupted longitudinal fascicles (arrows) and ectopic axon crossing of the CNS midline (arrowhead). (**F**) Graph quantifying midline crossing by Fas2-positive axons in *ftz$_{ng}$GAL4 UAS-Dscam1-30-30-2*, *NetA,B ftz$_{ng}$GAL4 UAS-Dscam1-30-30-2* and *NetA,B ftz$_{ng}$GAL4* embryos. There is no statistical difference in the amount of midline crossing observed when *NetA,B* is deleted (Poisson regression; n=15 for each category). (**G**) Wild-type embryo stained with MAb BP102. (**H**) Embryo carrying two copies of the *ftz$_{ng}$GAL4* and *UAS-Dscam1-30-30-2* transgenes stained with MAb BP102 showing disrupted separation of the anterior and posterior commissures (arrowhead), and disrupted longitudinal axon tracts (arrow).

These results confirm those obtained with BP102 staining, in that Dscam genes enhance the *fra* commissural axon phenotype (Fig. 4G). In addition, in *Dscam fra Dscam3* triple mutants, most SP1 axons cross the midline, suggesting the existence of additional attractive receptors. We also analyzed the effect of the *Dscam fra* double mutant on *egl*-positive commissural axons, which cross the midline midway through axonogenesis. *fra* mutants have no effect on the EG axon bundle (Garbe and Bashaw, 2007), but *Dscam fra* double mutants lead to 37% of EG axon bundles being thin or absent (n=112 segments). We chose to focus on SP1 axons as Fig. 4 clearly shows how follower axons can fasciculate with existing pathways. There are two examples of *egl* axons encountering a missing commissure and rerouting to the nearest intact commissure (Fig. 4J,K). This emphasizes the importance of analyzing pioneer axons to understand axon guidance phenotypes. Interestingly, SP1 cell body migration defects present in *fra* mutants are not enhanced by *Dscam* (Fig. 4H). However, we found that *Dscam* enhances *fra* cell migration defects in the salivary glands (see Fig. S3 in the supplementary material) to a penetrance greater than that seen in *Netrin* mutants (Kolesnikov and Beckendorf, 2005). The predominant phenotype observed was stalling of migration or kinking of the gland, which reflects growth in alternating directions. Dscam proteins therefore appear to be important for axon guidance and cell migration in different tissues.

Dscam overexpression induces ectopic midline crossing

As *Dscam* mutants cause a failure to cross the midline in combination with other mutations, we asked whether *Dscam* overexpression could cause axons to be attracted to the midline inappropriately. Pan-neuronal overexpression of *Dscam* with either *sca-GAL4* or *elav-GAL4* using the UAS-GAL4 system (Brand and Perrimon, 1993) did not yield detectable phenotypes. However, overexpression of *Dscam* by the *fushi tarazu* neurogenic (*ftz$_{ng}$*) promoter, which drives expression in longitudinal axons that do not normally cross the midline (Lin et al., 1994), resulted in ectopic axon crossing of the midline (Fig. 5B). Interestingly, the induced crossing was not suppressed by a *NetA* and *NetB* deficiency (Fig.

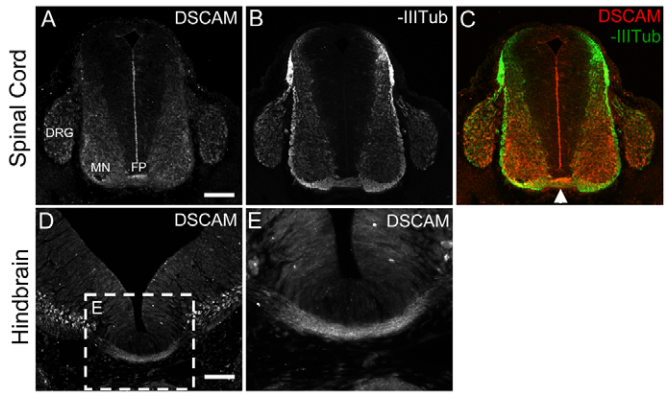

Fig. 6. DSCAM is expressed by commissural axons in the mouse CNS. Sections of embryos were labeled with antibodies against DSCAM and neuron-specific βIII-tubulin. (A-C) DSCAM is expressed in many neurons in the developing spinal cord. The protein is also detected in commissural axons. (**A**) Anti-DSCAM antibody labeling of E11.5 spinal cord, including motor column (MN) and commissural axons crossing the floor plate (FP). The dorsal root ganglia (DRG) are labeled as well. (**B**) The pan-neuronal β-Tubulin (β-IIITub) labelng of the same section shows the location of neurons. (**C**) Merge of A and B. DSCAM localizes to an inner subset of the commissural fibers (yellow, arrow). (D,E) DSCAM labeling of sections of rhombomere 1 of the E10.5 hindbrain. (**D**) Both neuron cell bodies and axons are labeled by anti-DSCAM. (**E**) A high magnification view of the boxed region in D shows strong DSCAM signal in the hindbrain commissure. Scale bars: 100 μm in A; 50 μm in D.

5D,F), suggesting that either Dscam is not responding to netrin proteins from the midline, or that Dscam can respond to more than one midline-derived attractive cue. Overexpression of multiple copies of each transgene leads to more severe phenotypes (Fig. 5E,H). Surprisingly, the initial projection of the pCC axon, which pioneers the medial Fas2-positive longitudinal affected by *Dscam* overexpression, was unaffected (*n*=220 axons, stages 12 and 13, one copy of ftz_{ng}-*GAL4* and *Dscam*). The complexity of the anti-Fas2 expression pattern after stage 13 makes it difficult to monitor the pCC axon projection, so we used *RN-GAL4* combined with a *UAS-tau-lacZ* reporter, which expresses specifically in pCC (and also two motoneurons) (Fujioka et al., 2003). Overexpression of *Dscam* by *RN-GAL4* had no effect on the pCC axon trajectory (*n*=242 axons) when assayed at stages 16/17. pCC does not express *fra* (Hiramoto and Hiromi, 2005), so we simultaneously overexpressed *Dscam* and *fra* using *RN-GAL4*, but the pCC axon did not cross the midline (*n*=211 axons). The results suggest that additional components will be required to generate a phenotype in pCC (see Discussion).

Dscam expression on mouse commissural axons

In situ hybridization studies have previously shown that although both Dscam and the related DscamL1 mRNAs have distinct expression patterns in the developing spinal cord, both expressed in the region where commissural neuron cell bodies lie (Yamakawa et al., 1998; Agarwala et al., 2001; Barlow et al., 2002). The evolutionarily conserved binding of netrin proteins and Dscam proteins suggested that Dscam might be important for midline crossing in vertebrates. To test whether Dscam could function as a Netrin receptor in vertebrates, we examined the distribution of mouse Dscam protein at the time of commissural axon growth in mice. Dscam protein was found to be present on axons known to be Netrin responsive (Shirasaki et al., 1996) in the hindbrain at E10.5, and on a subset of axons crossing the floorplate at E11.5 in the spinal cord (Fig. 6). There is expression in other regions, including DRG neurons, correlating with the *Dscam* mRNA expression pattern. The localization of Dscam protein to commissural axons in the mouse is consistent with an evolutionarily conserved role for Dscam as a Netrin receptor.

DISCUSSION

It is known that axons are attracted to the CNS midline by netrin proteins signaling through the DCC family of receptors. We have identified Dscam cell adhesion molecules as required for attraction to the midline in *Drosophila*. This was unexpected, as the role of Dscam proteins in mediating repulsion between neurites has been well characterized (Zipursky et al., 2006). In vertebrate retina formation, Dscam proteins have been shown to mediate attraction (Yamagata and Sanes, 2008) and repulsion (Fuerst et al., 2008), suggesting that like other axon guidance molecules, the output of Dscam proteins may be dependent on context, such as the presence or absence of co-receptors or downstream effectors. In vitro, Netrin proteins bind Dscam proteins in an evolutionarily conserved manner, with a similar affinity to the known Netrin receptor DCC. In vivo, *Dscam* and the Netrin genes genetically interact in a dose-sensitive manner in BN axon guidance. These data suggest that Dscam is a receptor required to mediate attraction to Netrin diffusing from the CNS midline and mesoderm.

The dramatic reduction in number of midline crossing axons in *Dscam fra* and *Dscam fra Dscam3* mutant combinations, with defects significantly greater than seen in *NetA,B* mutants, suggests that Dscam responds to additional ligands as well as to Netrin. In vertebrates, sonic hedgehog (Shh) has been identified as a netrin 1-independent chemoattractant for commissural axons (Charron et al., 2003). *Drosophila hedgehog* (*hh*) is required for the specification of the midline glia that commissural axons grow towards, but does not appear to be acting as a chemoattractant (Hummel et al., 1999b). Therefore, the missing midline chemoattractant(s) still remains to be identified, but we predict it will bind to Dscam genes. We favor the hypothesis that Dscam proteins participate in both Netrin-dependent and -independent pathways at the CNS midline.

A receptor role for Dscam was not entirely unexpected, as several groups had proposed the existence of a core molecular function for Dscam, independent of the diversity of protein isoforms (Wang et al., 2004; Zhan et al., 2004; Chen et al., 2006). Each group attempted to rescue *Dscam* mutant phenotypes by transgenic expression of a single *Dscam* isoform, and achieved partial rescue of their phenotype of interest. [For a discussion of the role of diversity in *Dscam* function, see Zipursky et al. (Zipursky et al., 2006).] Based on rescue of adult mechanosensory neurons, Chen et al. (Chen et al., 2006) hypothesized that the core function depends on a receptor-ligand interaction that may not engage the variable domains of the protein. Netrin binding could fulfill this role. As Netrin binding is evolutionarily conserved, we think that Netrin reception is likely to be independent of the *Dscam* diversity only found in insects. The ability of *Dscam* to mediate homophilic cell adhesion is also evolutionarily conserved (Agarwala et al., 2000; Wojtowicz et al., 2007; Yamagata and Sanes, 2008). Our results place Dscam in the small group of cell-surface molecules that can act as both receptors and mediate cell adhesion, as exemplified by the neural cell-adhesion molecule NCAM (Paratcha et al., 2003).

Molecular mechanisms of Dscam function

DCC is converted from an attractive receptor for Netrin to a repulsive receptor by heterodimerization with Unc5 (reviewed by Moore et al., 2007). This suggests that Dscam could be converted to an attractive receptor by interaction with other receptors. This model is supported by the trans-heterozygous interaction between *Dscam* and *fra* in BN, which places them in the same genetic pathway. Dscam is clearly not essential for Netrin signaling, in part because there are no *Dscam* homologs in *C. elegans*. However, using two different attractive Netrin receptors increases the potential complexity of responses to Netrin. Dscam proteins may also respond to additional cues, either alone or in combination with other receptors. The ectopic crossing induced by *Dscam* overexpression is significant, as *fra* overexpression alone is not sufficient to induce midline crossing (Dorsten et al., 2007). It is possible that Fra functions in a complex, and may require simultaneous overexpression of multiple components to generate a phenotype, as has been seen for γ-secretase activity (Edbauer et al., 2003). Interestingly, overexpression of *fra* lacking the entire cytoplasmic domain (*fraΔC*) can produce completely commissureless phenotypes, which are much stronger than *fra* mutant phenotypes (Garbe et al., 2007), suggesting that *fraΔC* could be inactivating a complex that responds to multiple attractive cues from the midline.

Organogenesis

Our phenotypic analysis of salivary glands demonstrates that *Dscam* can function in cell migration as well as in axon guidance. Humans with Down Syndrome (DS) frequently suffer from congenital heart disease, particularly atrioventricular septal defects (Yamakawa et al., 1998; Barlow et al., 2001). The developmental origin of these defects is probably disruption of directed growth of endocardial cushions, and *Dscam* is an excellent candidate to play a role in this process, especially if it can function as a receptor to guide migration. *Dscam* has also been associated with the mental retardation component of DS; *Dscam* and the related *DscamL1* gene are expressed in the cortex and cerebellum (Agarwala et al., 2001; Barlow et al., 2002) suggesting that *Dscam* may function in neuronal and axonal migrations during brain development. *Dscam* may be responsible for the abnormalities in intestinal formation and the associated enteric neuron defects seen in individuals with DS (Yamakawa et al., 1998). It should be noted that we have been analyzing loss-of-function phenotypes, and DS arises due to an additional copy of chromosome 21. Three copies of *Dscam* may increase basal levels of cell or axon migration, or may stimulate enhanced responses to external signals in any of the processes described above. We found it quite difficult to generate overexpression phenotypes of *Dscam*, suggesting that other components may be required for its function, and this may apply to individuals with DS too, limiting the tissues in which trisomy for *Dscam* could have an effect.

Conclusion

The evolutionary conservation of Dscam proteins may be explained by their dual ability to act as cell-adhesion molecules and as receptors. Dscam proteins provide a new starting point for fully understanding Netrin signaling, as well as for identification of additional axon guidance cues. Dscam-associated phenotypes in DS can be re-evaluated as a consequence of altered cell migration and axon guidance.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/135/23/3839/DC1

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